

# CONCANAVALIN A AND WHEAT GERM AGGLUTININ RECEPTORS ON *Dictyostelium discoideum*

## Their Visualization by Scanning Electron Microscopy with Microspheres

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The cellular slime mold, *Dictyostelium discoideum*, is a convenient model for studying cellular interactions during development. Evidence that specific cell surface components are involved in cellular interactions during its development has been obtained by Gerisch and co-workers (1, 2) using immunological techniques. Smart and Hynes (3) have shown that a cell surface protein can be iodinated on cells in aggregation phase, but not in vegetative phase, by the lactoperoxidase procedure. Recently, McMahon et al. (4), and Hoffman and McMahon<sup>1, 2</sup> have demonstrated, by SDS gel electrophoresis, considerable differences in cell surface proteins and glycoproteins of plasma membranes isolated from cells at different stages of development.<sup>3</sup>

Plant lectins have also been used to monitor changes in cell surface properties of *D. discoideum* cells during development. Weeks and co-workers (5, 6) have detected differences in the binding and agglutination of cells by concanavalin A (Con A). Gillette and Filosa (7) have shown that Con A inhibits cell aggregation and prematurely induces cyclic AMP phosphodiesterase. Capping of Con A receptors has also been reported (8). Reitherman et al. (9) have recently reported that agglutination

of cells by several plant lectins and the slime mold-agglutinin, discoidin, changes during development. Such studies indicate that differences in surface properties exist for cells at various stages of development. However, owing to the uncertainties in the factors which contribute to lectin-induced cell agglutination (10), the molecular basis for these observations remains to be determined.

In this study, we have used microspheres (11–14) coupled to either Con A or wheat germ agglutinin (WGA) as visual markers to study by scanning electron microscopy the topographical distribution of lectin receptors on *D. discoideum* cells fixed at different stages of development. We also describe the effect of labeling on the distribution of lectin receptors and on the morphology of the cell surface.

## MATERIALS AND METHODS

Concanavalin A (3× crystallized) and WGA were purchased from Miles Laboratory, Kankakee, Ill. Di-*N*-acetyl chitobiose was a generous gift of Dr. B. T. Shier, The Salk Institute for Biological Studies, La Jolla, Calif.;  $\alpha$ -methyl-mannoside was obtained from Sigma Chemical Co., St. Louis, Mo. Copolymer methacrylate microspheres of average diameter 35 nm or 100 nm (14) were a generous gift of Doctors A. Rembaum and S. P. S. Yen. Phosphate-buffered saline was prepared as previously reported (11).

*D. discoideum* strain AX-3 was grown in 1.6× HL-5 medium at 22°C on a rotary shaker and harvested from cultures in log phase of growth. Developing cells were prepared by plating out cells on Whatman filter paper as described previously (4, 14, 15).

## Preparation of Lectin-Microsphere Conjugates

Fluorescent microspheres were prepared by reacting 7.8 mg of fluorescein isothiocyanate with 10 ml of microspheres substituted with diaminoheptane (15 mg/ml) in 0.1 M carbonate buffer, pH 10 for 12 h at 25°C. Lectin-

<sup>1</sup> Hoffman, S., and D. McMahon. 1976. The role of the plasma membrane in the development of *Dictyostelium discoideum*. II. Developmental and topographical analysis of polypeptide composition. Manuscript submitted for publication.

<sup>2</sup> Hoffman, S., and D. McMahon. 1976. The role of the plasma membrane in the development of *Dictyostelium discoideum*. III. Developmental analysis of glycopeptide composition. Manuscript submitted for publication.

<sup>3</sup> McMahon, D., M. Miller, and S. Long. 1976. The role of the plasma membrane in the development of *Dictyostelium discoideum*. I. Purification of the plasma membrane of *Dictyostelium discoideum*. Manuscript submitted for publication.

microsphere conjugates were prepared by the two-step glutaraldehyde procedure (12, 13). In the coupling reaction, glutaraldehyde-activated microspheres (15 mg/ml) were stirred in the presence of WGA (0.5 mg/ml) or Con A (1 mg/ml) in 0.01 M phosphate buffer pH 7.0 for 24 h at 25°C. Lectin-microsphere conjugates were separated from uncoupled lectin as previously described (13).

### Labeling of Cells

All labeling experiments were performed at 25°C. Cells plated on filters were labeled by suspending a 6-mm × 6-mm piece of the filter in 50  $\mu$ l of either Con A-microsphere or WGA-microsphere conjugate. At various times, the filters were rinsed in phosphate-buffered solution (PBS). In control experiments,  $\alpha$ -methyl mannoside or *N*-acetyl chitobiose was present during labeling or added after labeling. In some experiments, cells at aggregation (12 h) or pre-culmination (18 h) stage were washed off the filters with cold buffer and dissociated by pipetting the suspension through a 5-ml pipette 10 times. The dissociated cells were replated on filter paper and labeled as described above. Cells in suspension were labeled by incubating  $10^6$  cells, in 50  $\mu$ l of conjugate for the desired time. Labeled cells were then separated from unbound conjugate by repeated centrifugation at 400 g.

In some experiments, cells at various stages of development were fixed with 0.25% glutaraldehyde in PBS for 30 min at 25°C before labeling. Excess glutaraldehyde was removed by rinsing the cells in PBS and incubating the cells for 1 h in PBS containing 0.1 M glycine. The cells were then labeled with the conjugate.

Double-labeling experiments were carried out as follows. Unfixed cells were labeled with Con A-microsphere conjugates for 40 min at 25°C. After the unbound conjugate was removed by washing, the cells were fixed in 0.25% glutaraldehyde-PBS and treated with glycine as described above. The labeled fixed cells were then treated with a second conjugate in the presence or absence of inhibitor. After 60 min, the cells were again washed free of excess conjugate and prepared for scanning electron microscopy (SEM).

### Preparation of Cells for SEM

Cells were fixed for 1 h with 1.25% glutaraldehyde in PBS at 25°C immediately after labeling. Cells dehydrated in ethanol were critical-point dried with Freon 13 and prepared for SEM as previously reported (11). The samples were examined on an ETEC Autoscan scanning electron microscope operated at 20 kV.

## RESULTS

### Distribution of Lectin Receptors on Prefixed Cells

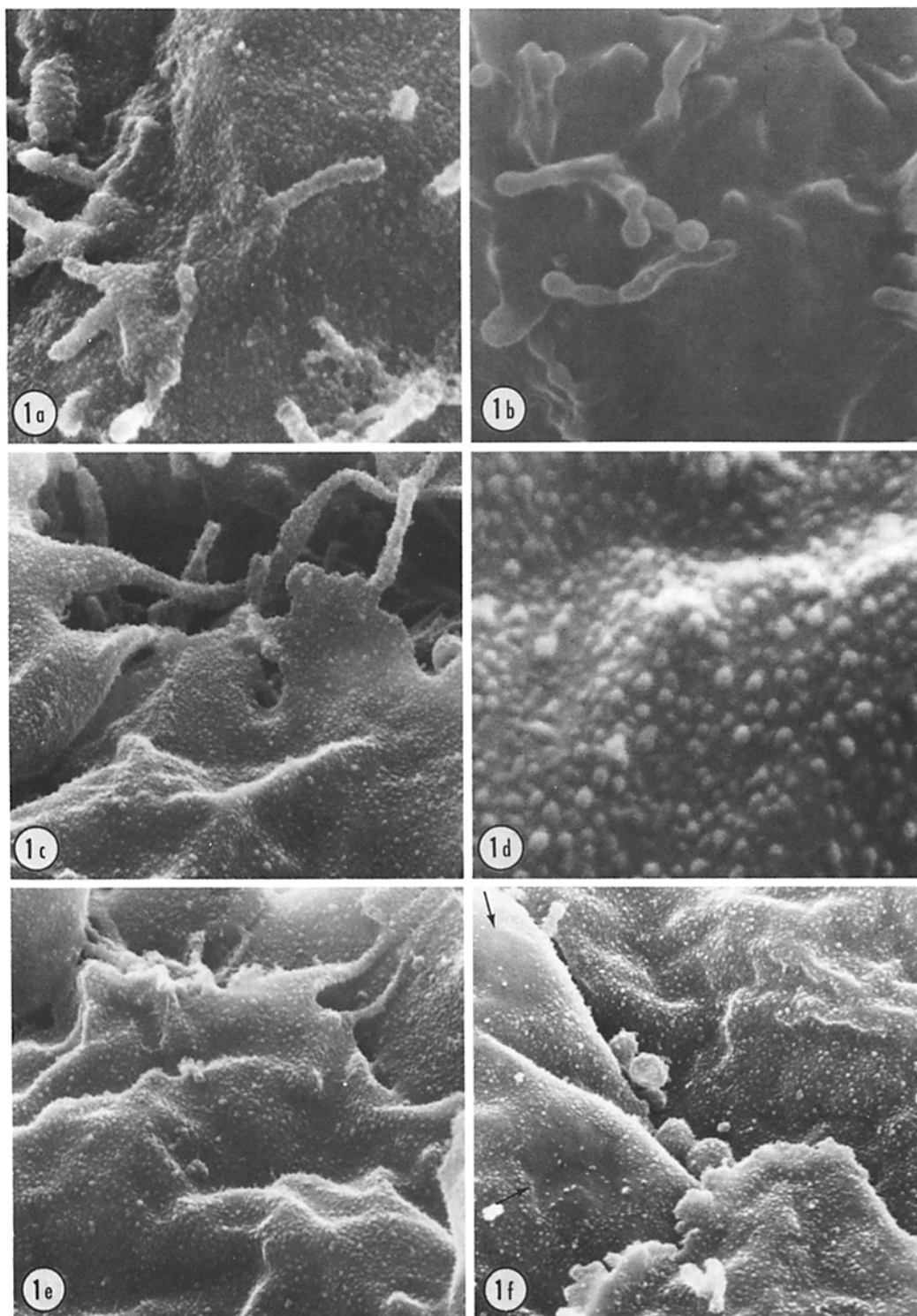
In the initial series of experiments, cells were fixed with glutaraldehyde before labeling. As

shown in Fig. 1*a*, cells fixed in suspension during growth phase and subsequently labeled with Con A-microsphere markers (8 mg/ml) exhibited a dense, uniform distribution of microspheres on their surface including their microvilli. As expected from previous results, cells incubated in the presence of  $\alpha$ -methyl mannoside exhibited only a few microspheres per cell (Fig. 1*b*), but addition of  $\alpha$ -methyl mannoside to prelabeled cells only partially reversed the binding of the Con A conjugates. 10 min after the addition of 0.05 M  $\alpha$ -methyl mannoside, approximately 30% of the microspheres remained bound to the cells; after 1 h, only 3% of the microspheres were seen on the cells. Cells plated on filters and fixed after 15 min, 6 h, or 12 h of development showed the same dense, uniform distribution of markers observed for cells in growth phase. This is exemplified by Fig. 1*c*, *d* for *D. discoideum* cells which have been allowed to develop on filters for 6 h and by Fig. 1*e* for 12-h cells. Markers are present uniformly on the exposed surface and in areas of cell contact. After 18 h, cells from pseudoplasmodia showed both areas of heavy labeling and areas free of markers (Fig. 1*f*). Occasional cells showed extensive patches of surface which were free of microspheres.

Glutaraldehyde-fixed *D. discoideum* cells labeled with WGA-microsphere conjugates, at all stages of development, displayed the markers in a tightly packed array at saturating concentrations of reagent. A representative result is shown in Fig. 2*a* for cells plated on filters for 6 h. In control experiments, the binding of WGA-microsphere markers was inhibited by *N*-acetyl chitobiose (Fig. 2*b*) and was reversed by addition of this inhibitor to labeled cells.

### Labeling of Lectin Receptors on Unfixed Cells

Unfixed cells were treated with lectin-microsphere conjugates for various times in order to determine the effect of these reagents on cell surface morphology and receptor distribution. As shown in Fig. 3*a*, cells plated on filters for 15 min and then treated with Con A-microsphere conjugates for 10 min transformed from a flattened, irregular shape to a spherical geometry. The microvilli were no longer randomly distributed on the cell surface; microvilli and other surface projections were free of markers (Fig. 3*b*). After labeling for 20 min, tight clusters of shortened



**FIGURE 1** Scanning electron micrographs of *D. discoideum* cells fixed with 0.25% glutaraldehyde at different stages of development and treated with Con A-microsphere markers with an average diameter of 35 nm. (a) Growth phase cell displaying dense, uniform distribution of markers on its cell surface and microvilli; (b) growth phase cell treated with Con A-microspheres in the presence of 0.05 M  $\alpha$ -methyl mannoside; only a few microspheres bind to the smooth cell surface; (c) cells plated on filters and allowed to develop for 6 h at 22°C show markers covering the contoured cell surface and microvilli; (d) same as in (c) but viewed at a higher magnification; (e) cells allowed to develop for 12 h (aggregation stage) exhibiting markers on the cell surface and areas of cell-cell contact; (f) 18-h cells in slug or preculumination stage showing areas of dense labeling adjacent to areas (arrow) free of markers. (a)  $\times 15,000$ ; (b)  $\times 15,000$ ; (c)  $\times 12,000$ ; (d)  $\times 52,000$ ; (e)  $\times 12,000$ ; (f)  $\times 10,000$ .

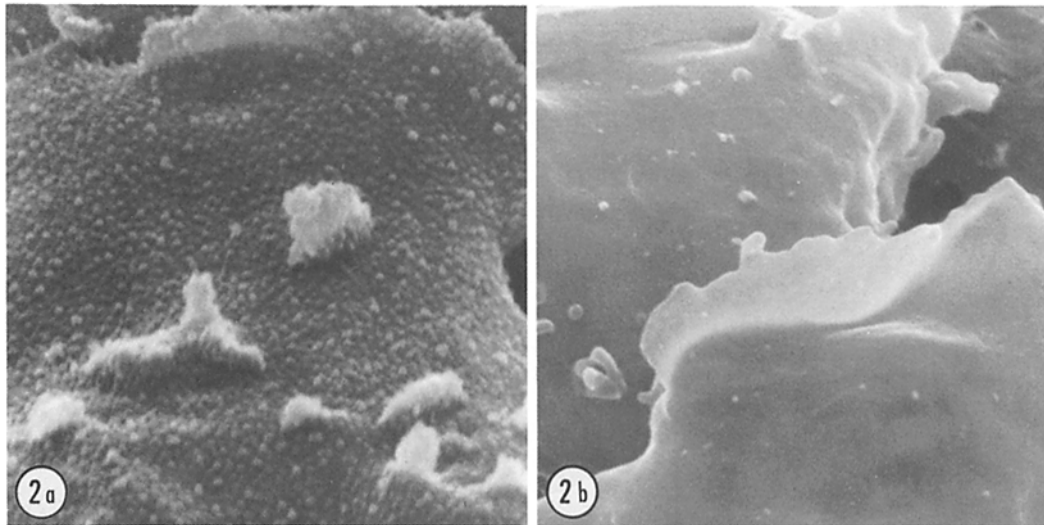


FIGURE 2 *D. discoideum* cells fixed with 0.25% glutaraldehyde after 6 h of development. (a) Cells treated with WGA-microspheres exhibit a dense uniform distribution of microspheres (average diam 35 nm) on the cell surface and surface projections (b) control: cells treated with WGA-microspheres in the presence of 0.01 M *N*-acetyl chitobiose display only a few markers on their surface (a)  $\times 19,000$ ; (b)  $\times 14,000$ .

microvilli were found on a small area of the cell (Fig. 3c). Approximately 50% of the cells exhibited this cluster. The microspheres were still arranged in patches. As depicted in Fig. 3d–e, cells labeled for 40 min and 60 min showed distinct clusters of microvilli protruding from the cell. The clusters were predominantly at areas of cell-cell contact (Fig. 3d) or cell-substrate (filter) contact (Fig. 3e). Long filaments present in these contact regions extended from the cell to the adjacent cell or the filter. Con A-microspheres were localized in a cap which surrounded the cluster of microvilli (Fig. 3d–e). Note also that the microspheres are not found on the microvilli which form the morphological caps; however, microspheres were seen on the long filaments which extended from cell to cell or cell to filter. After 2 h of incubation with Con A-microspheres, most cells were smooth, free of microvilli and sparsely labeled with the conjugates (Fig. 3f). Occasionally, clusters and other cell debris labeled with microspheres were observed to be linked to a cell by a long filamentous extension of the cell. These changes in cell surface topography and distribution of Con A-microspheres also occurred with cells which had developed for 12 or 18 h.

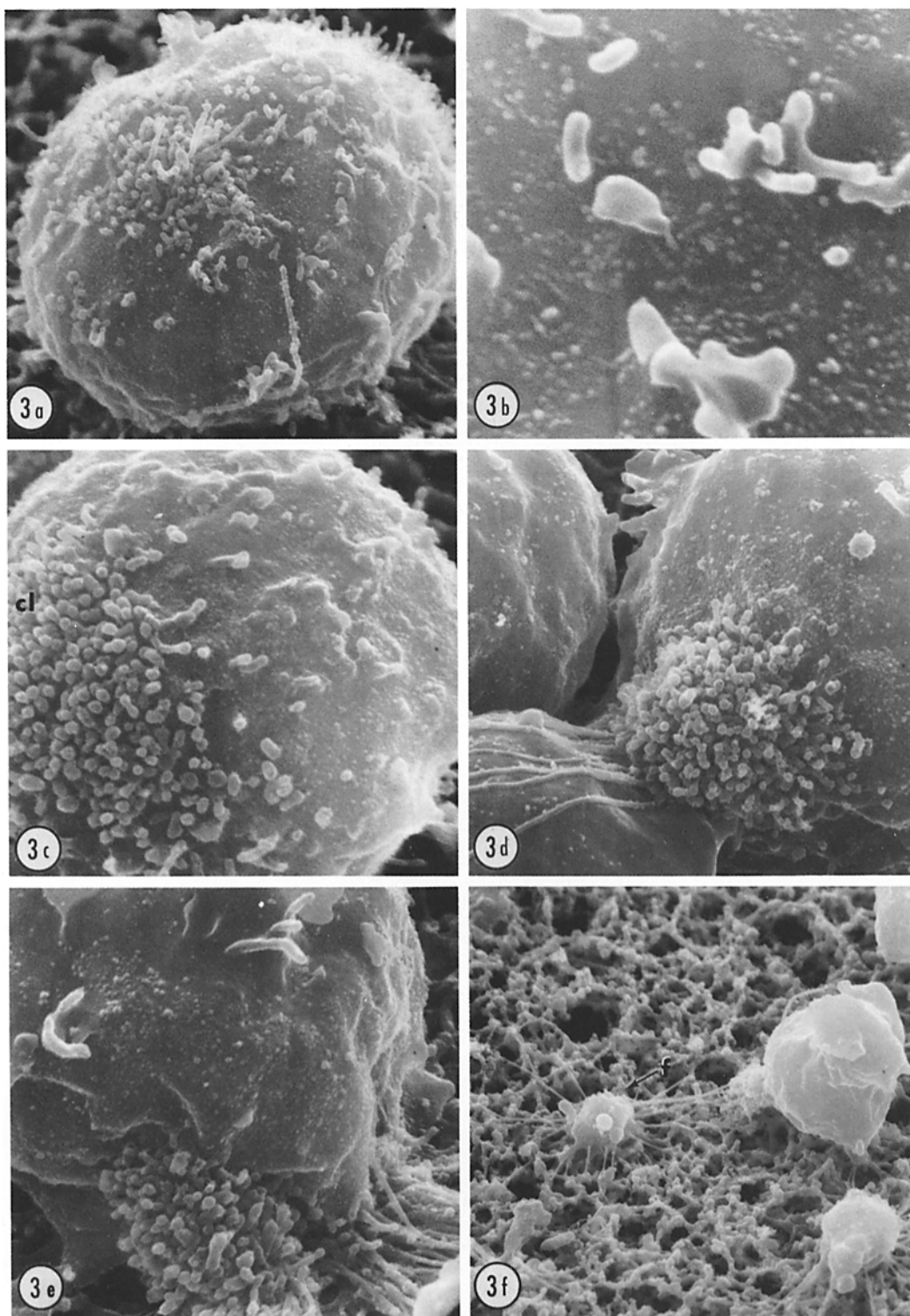
To determine whether these observed cell surface changes were a result of specific interaction with Con A or due to other conditions to which

the cells were exposed during labeling, cells were treated in parallel with either Con A-microsphere conjugate in the presence of 0.05 M  $\alpha$ -methyl mannoside, 0.5 mg/ml free Con A, or PBS buffer alone. Cells treated at 25°C with PBS and with Con A conjugate in the presence of its inhibitor exhibit the same flattened cell surface morphology as cells plated on filters at 22°C. On the other hand, cells incubated with free Con A showed similar cell surface changes found when Con A-microsphere conjugates were used. Cells labeled in suspension with fluorescein-tagged microspheres for 40 min and examined with a fluorescence microscope showed caps concentrated in regions of cell contact (Fig. 4).

Labeling of *D. discoideum* cells with WGA-microspheres for various lengths of time and at different stages of development had no effect on cell surface features. Markers were arranged in a dense uniform array over the entire cell; did not cap; and the distribution of microvilli did not change noticeably.

#### *Sequential Labeling with Con A and WGA-Microspheres*

The difference in behavior of Con A and WGA-microsphere markers observed with unfixed cells suggested that at least some WGA receptors are



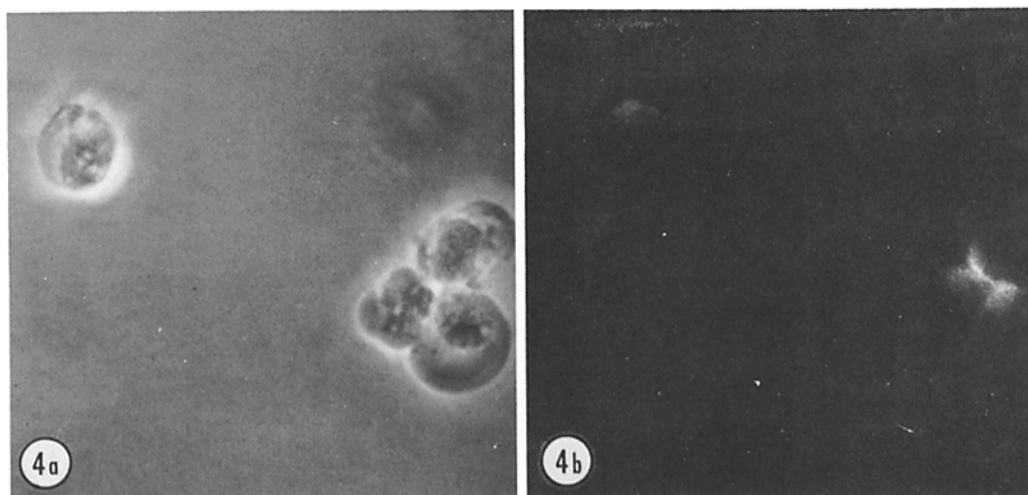


FIGURE 4 Cells which had been labeled in suspension for 40 min with fluorescent Con A-microspheres; (a) visualization by phase contrast; (b) visualization by fluorescence. Note that caps of the Con A-microspheres commonly face toward the center of aggregates of cells.  $\times 1,000$ .

located on different components of the membrane than are Con A receptors. To test this hypothesis, cells were labeled with Con A-microspheres, then fixed in glutaraldehyde, and finally relabeled with either Con A- or WGA-microsphere conjugates. When Con A-labeled cells were relabeled with Con A-microspheres of either 100 nm or 35 nm diameter, the patchy distribution of marker seen after the initial labeling (Fig. 3 *d-e*) prevailed. Fig. 5 *a* shows that, as expected, only a few 100-nm markers were bound to the cells; microvilli and the clusters of microvilli were generally not labeled after this treatment. However, when cells were challenged with WGA-microspheres, a dense, uniform distribution of microspheres was observed over the entire cell and on the microvilli (Fig. 5 *b*).

In control experiments, cells relabeled with WGA-microspheres in the presence of *N*-acetyl chitobiose were not uniformly labeled, but showed the distribution of scattered and rare microspheres over the majority of the cell, just as was seen after the initial labeling with Con A-microspheres. The results of this sequential labeling were the same for cells which were allowed to develop for 15 min or 18 h before the double-labeling experiment was performed.

#### DISCUSSION

Our results, that Con A and WGA microsphere markers are distributed in a dense, uniform array over the entire surface, including the microvilli, of glutaraldehyde-fixed *D. discoideum* cells in vege-

FIGURE 3 The effect of continuous labeling with Con A-microspheres on the morphology of the cell surface and distribution of Con A receptors. Unfixed cells, which had been plated out on filters for 15 min, were treated with the Con A-conjugates for varying lengths of time at 25°C. (a) After 10 min of labeling, cells became more spherical in shape and many of the microvilli were located within a small area of the cell surface; (b) higher magnification which shows that the microsphere markers were distributed in patches on the cell surface, but were absent on the microvilli; (c) after 20 min, the microvilli which localized in a tight cluster (*d*) are shorter in length and often bulbous in appearance, and markers are still distributed in patches over the entire cell surface; (d) after 40 min, the markers were concentrated in a cap surrounding the cluster: in the area of cell contact, a cluster formation can be seen with long filaments interacting with the smooth surface of the adjacent cell; (e) after 60 min, the cluster formation, seen in contact with the filter, protrudes outward from the cell surface, and markers are still localized near the cluster; (f) after 2 h, the cells appear smooth, sparsely labeled with markers and cell fragments (*f*), labeled with microspheres, are seen. (a)  $\times 8,400$ ; (b)  $\times 30,000$ ; (c)  $\times 11,000$ ; (d)  $\times 14,000$ ; (e)  $\times 10,000$ ; (f)  $\times 4,600$ .

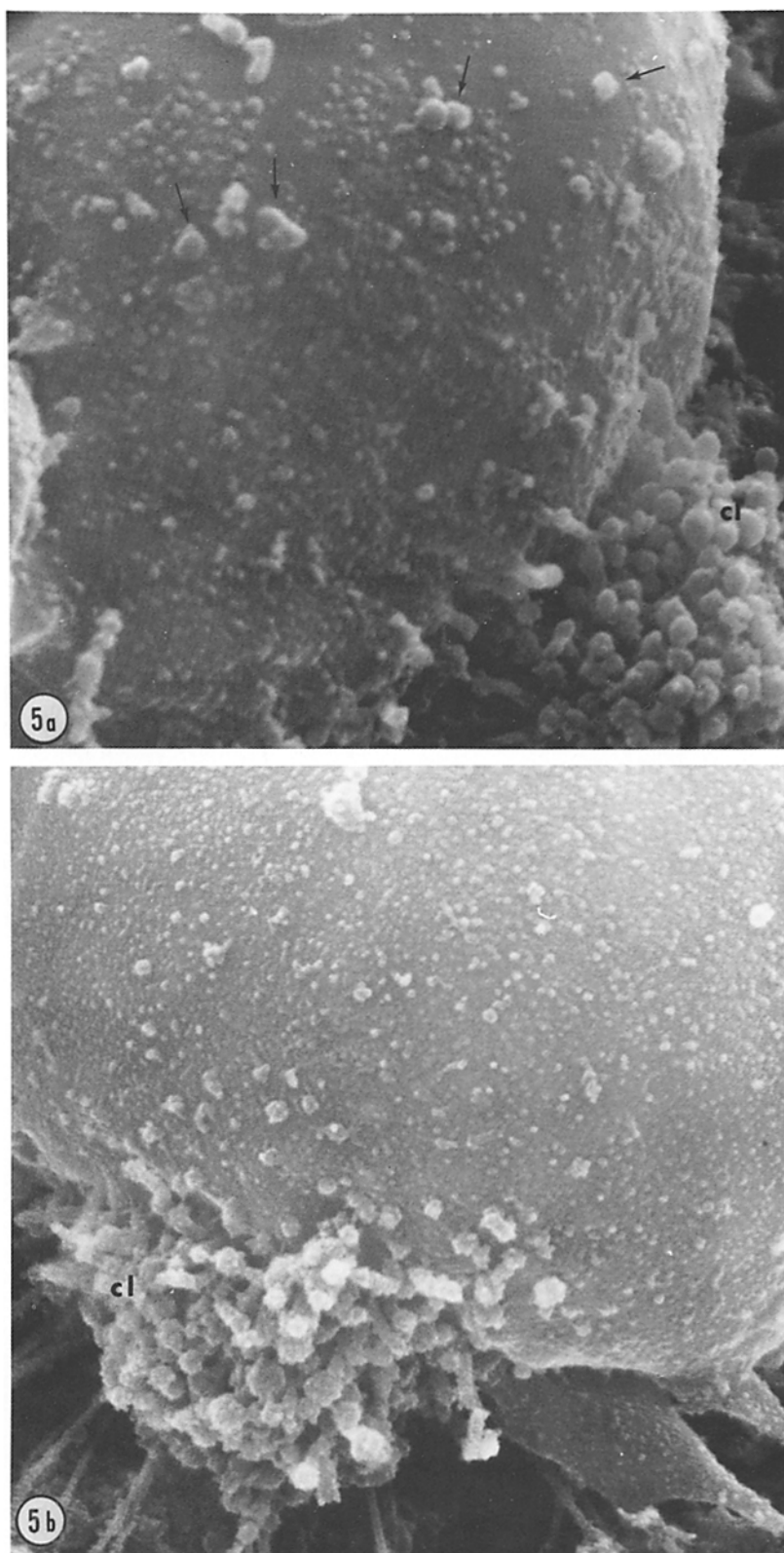


FIGURE 5 Sequential labeling of lectin receptors on *D. discoideum* cells which have been plated on filters for 15 min, labeled with Con A-microspheres (35 nm diam) for 35 min at 25°C, washed in buffer, and fixed with 0.25% glutaraldehyde. (a) Relabeled with Con A-microspheres (110 nm diam). Only a few 110-nm markers (arrows) bind to the cell; (b) relabeled with WGA-microspheres 35 nm in diameter. In contrast to those in (a), the markers in (b) cover the entire cell surface and also bind to the cluster of microvilli (cl). (a)  $\times 22,500$ ; (b)  $\times 15,000$ .



tative (Oh) and aggregation stage (6–12 h), indicate that previously reported differences in the agglutination of *D. discoideum* by Con A (5) or WGA (9) are not caused by gross differences in the arrangement of lectin-binding sites. Over  $4 \times 10^5$  binding sites for each lectin per *D. discoideum* cell can be resolved with these visual markers.

A notable feature in the labeling pattern of Con A-microspheres was found on *D. discoideum* cells allowed to develop for 18 h. When glutaraldehyde-fixed cells were labeled with Con A-microspheres, bare areas were seen on some cell surfaces (Fig. 3f). This labeling pattern may reflect a different topographical distribution of Con A receptors on preculmination cells. While such a pattern might be simply an artifact resulting from the presence of patches of slime covering parts of the cell at this phase of development, we believe that this is not the case since we have found that the microspheres stick nonspecifically to the slime sheath of the preculminate and that  $\alpha$ -methyl mannoside does not prevent them from binding. The observation of patches free of Con A receptors might also have been the result of inadvertent fragmentation of cell groups during processing, resulting in exposure of potentially unlabeled areas of contact between the cells. This explanation also appears to be unlikely since equivalent bare areas were not seen with WGA-microspheres whose diameter equaled that of the Con A microspheres. This result, if supported by additional work, is of some interest since a theory for positional specification of developing cells (16) postulates an anisotropy of plasma membrane receptors.

Unfixed cells show distinct differences in the distribution of labeled WGA and Con A cell surface sites as a function of time. Whereas WGA-microspheres continued to be distributed in a tightly packed mosaic over the entire cell surface, Con A microspheres were redistributed into patches on the cell surface, and disappeared from the microvilli. The redistribution of labeled Con A receptors into patches is characteristic of a number of cell types (17–19).

As previously reported by Gillette et al. (8) for *D. discoideum* using the peroxidase staining technique, receptors labeled with Con A migrate to one pole of the cell form a caplike structure. The SEM has shown that associated with this redistribution of Con A receptors is a rounding up of the cells and a clustering of microvilli at the same end of the cell, a result which has not been previously

reported in any system. Spudich and coworkers (20, 21) have shown that actin filaments are present in *D. discoideum* cells and are associated with the cytoplasmic side of the plasma membrane. These structures may interact directly or indirectly with membrane components containing Con A-binding sites and serve to move them to one pole of the cell. Whether the bulbous microvilli and long filaments which are associated with the cap of receptors are “capped” by the same system or whether these morphological features arise *de novo* at the site of the cap cannot be determined from the evidence which is presently available. Such surface structures, however, appear to be involved in the Con A-induced cell aggregation as shown by the microscopic techniques described in this paper.

Finally, sequential labeling studies indicate that many, if not all, WGA receptors do not comigrate with labeled Con A receptors. This provides evidence that at least some of the surface receptors for these two lectins reside on different membrane components.

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